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（発達過程ラット網膜における trkB, low-affinity NGF receptor mRNA の局在）

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Localization of trkB and low-affinity NGF receptor mRNA in the developing rat retina

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Summary

The localization of trkB and low-affinity nerve growth factor receptor (LNGFR) mRNAs in the developing rat retina was examined by in situ hybridization. TrkB mRNA was expressed in the ganglion cell layer (GCL), in the inner border of the neuroblastic layer (NBL), and the inner border of the inner nuclear layer (INL). LNGFR mRNA was expressed in the GCL, in almost full thickness of the NBL, and in the intermediate part of the INL. Althogh both trkB mRNA and LNGFR mRNA were expressed in the GCL, expression pattern was different between these mRNAs; trkB mRNA was expressed in almost all cells in the GCL uniformly and the expression of LNGFR mRNA varied greatly from cell to cell. In addition, the expression of both mRNAs, especially LNGFR mRNA seemed to be down-regulated at P7, when programmed cell death of the RGCs was prominent. These observations indicate that LNGFR may modulate the function of trkB and that trkB and LNGFR play important roles in the development and maintenance of the RGCs.

Brain-derived neurotorophic factor (BDNF) supports the survival of rat and chick retinal ganglion cells (RGCs) in culture [9]. Furthermore, the timing of BDNF dependency coincides with the time of target innervation, and BDNF is expressed in the superior colliculus, the target tissue of optic nerve fibers[19]. These findings suggest that BDNF is the major neurotrophic factor for the development of the optic nerve fibers. BDNF binds to two kinds of transmembrane glycoproteins, low-affinity NGF receptor (LNGFR) and TrkB [17]. TrkB is a receptor tyrosine kinase encoded by the trkB proto-oncogene. Recent studies indicate that TrkB is the relevant receptor for BDNF [10,11]. LNGFR binds all neurotrophins with similar affinity but does not seem to be a functional receptor in the absence of a Trk receptor [2]. We have previously demonstrated by Northern blot analysis that trkB and LNGFR mRNAs in rat retina vary dramatically during perinatal days, that is, the period of synapse formation and programmed cell death of the RGCs [20]. To investigate roles of BDNF and it's receptors in the development of optic nerve fibers, we have examined the localization of trkB and LNGFR mRNAs in the developing rat retina by in situ hybridization.

Wistar albino rats were used in all dissections, and they were kept in darkness at night and illuminated during the day by incandescent lights. Timed-pregnant rats were killed by decapitation under ether anetsthesia, and embryos were quickly collected. Vaginal plug positive is recorded as embryonic day zero (E0). Parturition is between E21 and E22, taken as postnatal day zero (P0). Eye balls of pre- (E17) and postnatal (P0, 7, 14, 28) rats were removed, immersed in Optimum Cutting Temperature (O.C.T.) compound (Miles Inc.) and immediately frozen on dry ice. Mid-sagittal cryostat retinal sections (10μm) were collected onto poly-L-lysine (50 μg/ml)-coated slides . Each slides were hybridized to a ^{35}S-CTP-labeled LNGFR or trkB cRNA probe as described in the figure 1. legend.

After hybridization with an antisense RNA probe complementary to the transmembrane domain of the rat trkB, a positive hybridization signal was chiefly observed over cell bodies in the inner margin of the neuroblastic layer (NBL) at E17 (Fig.1,2-A). The ganglion cell layer (GCL) was not developed at this stage, although some cells seemed to begin to be separated from the NBL. At P0, the GCL became evident and almost all the cells in the GCL expressed trkB mRNA uniformly (Fig.1,2-B). The hybridization signal for trkB mRNA in the GCL seemed to be decreased from P0 to P7, because thickness as well as intensity of signal were decreased (Fig.1,2-B,C). This decrease of the hybridization signal corresponded to the result of the RNA blot analysis which demonstrated down-regulation of trkB mRNA from P0 to P7 in the whole retina [20]. At P7, the outer plexiform layer was now apparent as a slight separation of the nuclei of the NBL, of which inner half becomes the inner nuclear layer (INL). The signal for trkB mRNA became localized in the inner region of the INL (Fig.1,2-C). At P14, the signal for trkB mRNA in the GCL seemed to be increased, althogh cell number in the GCL remarkably decreased (Fig.1,2-D). Localization of trkB mRNA remained in the GCL and the inner region of the INL until P28 (Fig.1,2-E). In contrast, the sense probe did not show any specific labeling (Fig.1-K). Hybridization with a rat antisense LNGFR mRNA probe revealed a different expression pattern from that with a rat antisense trkB mRNA probe. At E17, a positive hybridization signal was chiefly observed over cell bodies in the inner region of the NBL. But, it was more diffusely ditributed within the NBL than that with trkB probe (Fig.1,2-F). At P0, the hybridization signal became more intense and was observed in almost full thickness of the NBL. The cells in the GCL also expressed LNGFR mRNA, of which signal varied greatly from cell to cell (Fig.1,2-G). The signal for LNGFR mRNA in the GCL remarkably decreased at P7 and only a small population of the RGCs

seemed to be positive for the LNGFR mRNA. The signals in the INL became localized in the intermediate portion of the INL (Fig.1,2-H). After P14, the localization of LNGFR mRNA remained to be in the GCL and the intermediate portion of the INL (Fig.1,2-I,J). In contrast, the sense probe did not show any specific labeling (Fig.1-L).

At early embryonal stages, retinal cells are mitotic and compose thick neuroblastic layer. During development, these cells differentiate into three layers; the outer nuclear layer, inner nuclear layer and the ganglion cell layer [16]. The INL contains the cell bodies of the bipolar cells, horizontal cells, amacrine cells and Müller cells. The bipolar cells are the second order neurons in the retina, which connects photoreceptors with the ganglion cells. The cell bodies of these cells lies in the intermediate portion of the INL. The horizontal cell and the amacrine cell are interneurons. Cell bodies of the former form the outer regions of the INL, and those of the latter form the inner region of the INL. Müller cells are glial cells which occupy the full thickness of the retina from the internal to external limiting membranes. The nuclei of these cells are found almost exclusively in the intermediate portion of the INL. The GCL contains the large cell bodies of RGCs [16]. Because signals for trkB mRNA were evident in the GCL and the inner portion of the NBL or the INL, trkB mRNA seems to be expressed in the RGCs and amacrine cells. Jelsma et al. demonstrated by in situ hybridization and immunohistochemistry that trkB expression in adult rat retina was concentrated in the RGCs and, probably, amacrine cells [8]. Ernfors et al. reported that mRNAs for trk, trkB, trkC are expressed in the GCL and neuroepithelium of the rat embryo [6]. Signals for LNGFR mRNA were localized in the entire region of the NBL from E17 to P0 and in the intermediate portion of the INL from the P7 to P28 in addition to the GCL. Therefore, LNGFR may be expressed in bipolar cells or Müller cells in addition to RGCs after P7. Immunohistochemical studies in developing rat

retina showed that NGFR was mainly localized in the GCL, and that less intense immunoreactivity was observed in the INL, especially Müller cells [22]. In the developing chick retina, in situ hybridization study revealed that NGFR was expressed in the GCL and the INL [7].

Although both of trkB and LNGFR are expressed in the GCL, their expression pattern was different from each other. Signals for trkB was observed in almost all the cells in the GCL rather evenly. In contrast, intensity of the signals for LNGFR mRNA varied from cell to cell. Because LNGFR is proved to modulate signal transduction through Trk [21], it is reasonable to speculate that the function of trkB is controlled by the amount of LNGFR. Difference of the localization in the INL between trkB and LNGFR also suggests their functional dissociation. In this area, the possibility remains that LNGFR acts as a co-factor of another tyrosine-kinase receptor (for example trkA) rather than trkB, or that it is an unknown functional factor such as an anchor for neurotrophins.

One of the major reasons why programmed cell death during neuronal development take place is that the amount of the trophic factors within the target area of their axon is limited. That is, during the period of synapse formation, neurons that could get trophic factors from their target would survive, while those could not would die [14]. In the development of visual system of albino rats, the optic nerve fibers first reach their targets, superior colliculus and lateral geniculate body at E17 [1], and the first synapses to be formed in their targets appear also at E17 [13]. Furthermore, the number of optic nerve fibers and RGCs decrease dramatically during the period from E20 to P7 [15]. Our data revealed that the number of RGCs slightly decreased at P7 and decreased in half from P7 to P14. We have previously shown by Northern blot analysis that trkB and LNGFR mRNAs in rat retina increase from E14 to E17 and dramatically decreased from P1 to P7 [20]. The present data also showed that trkB and LNGFR, especially

LNGFR, was down-regulated at P7 in the RGCs. Therefore, it is reasonable to speculate that this down-regulation of trkB and LNGFR mRNAs is related to the programmed death of the RGCs.

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Figure ledends

Figure 1

Localization of trkB mRNA (A~E) and LNGFR mRNA (F~J) in rat retina. Mid-sagittal sections were hybridized in situ with ^{35}S-labeled anti-sense RNA probe to trkB and LNGFR. (A)(F) : E17, (B)(G) : P0, (C)(H) : P7, (D)(I) : P14, (E)(J) : P28 ; (K)(L) : Mid-sagittal sections were hybridized with sense RNA probe at P0.; GCL=ganglion cell layer, INL=inner nuclear layer, ONL=outer nuclear layer, Scale bar=100 μm.

A 476-bp PCR fragment of rat trkB and a 497-bp fragment of rat LNGFR cDNA were subcloned into plasmid pBluescript SK (Stratagene) as described previously [20]. A probe for trkB we used recognizes both of full-length and truncated trkB. According to our previous results, however, full-length trkB are predominantly expressed in the developing rat retina. The plasmids were linearized and the cloned DNAs were transcribed in vitro by using T3 (antisense) or T7 (sense) RNA polymerase (Stratagene) in the presence of [a-(^{35}S)thio] CTP (NEN 1000 Ci /mmol;1 Ci=37Gbq). After fixation with 4% formaldehyde in phosphate buffer saline (PBS) for 10 minutes, slides were rinsed twice with PBS and once with deionized, autoclaved-water. Slides were then rinsed with 0.1M triethanolamine (pH 8.0), immersed in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes, dehydrated and air-dried. In situ hybridization was performed in a buffer contaning 50% formamid, 2 x SSC ,10mM Tris-Cl (pH7.4), 1 x Denhard's solution, 10% Dextran Sulfate, 0.2% SDS, 0.1M dithiothreitol, 0.25 mg/ml yeast tRNA, 0.5mg/ml salmon sperm DNA, ^{35}S-labeled cRNA probe of 1.0 x 10^5 cpm/μl overnight at 42°C. The sections were washed in 2 x SSC for 1 hour at room temperature and then in 2 x SSC for 1 hour at 60°C. They were immersed in 10 mM Tris-Cl (pH 7.4), 1mM EDTA (pH 8.0), 0.5M NaCl, 10μg/ml RNaseA for 30 minutes at

37°C, and then washed in 0.1 x SSC for 1 hour at room temperature, and in 0.1 x SSC for 1 hour at 65°C subsequently. They were cooled to room temperature, dehydrated and air-dried. Radioactivities of each slides were defined using an Image analyzer (FUJIX), and then dipped in Kodak NTB-2 emulsion and exposed for 5 weeks .

Figure 2

Brightfield image of in situ hybridization in rat retina. Counterstained with Hematoxyline blue. trkB (A~E), LNGFR (F~J); (A)(F) : E17, (B)(G) : P0, (C)(H) : P7, (D)(I) : P14, (E)(J) : P28 ; Abbreviation as per Figure1. Scale bar= 100 μm.

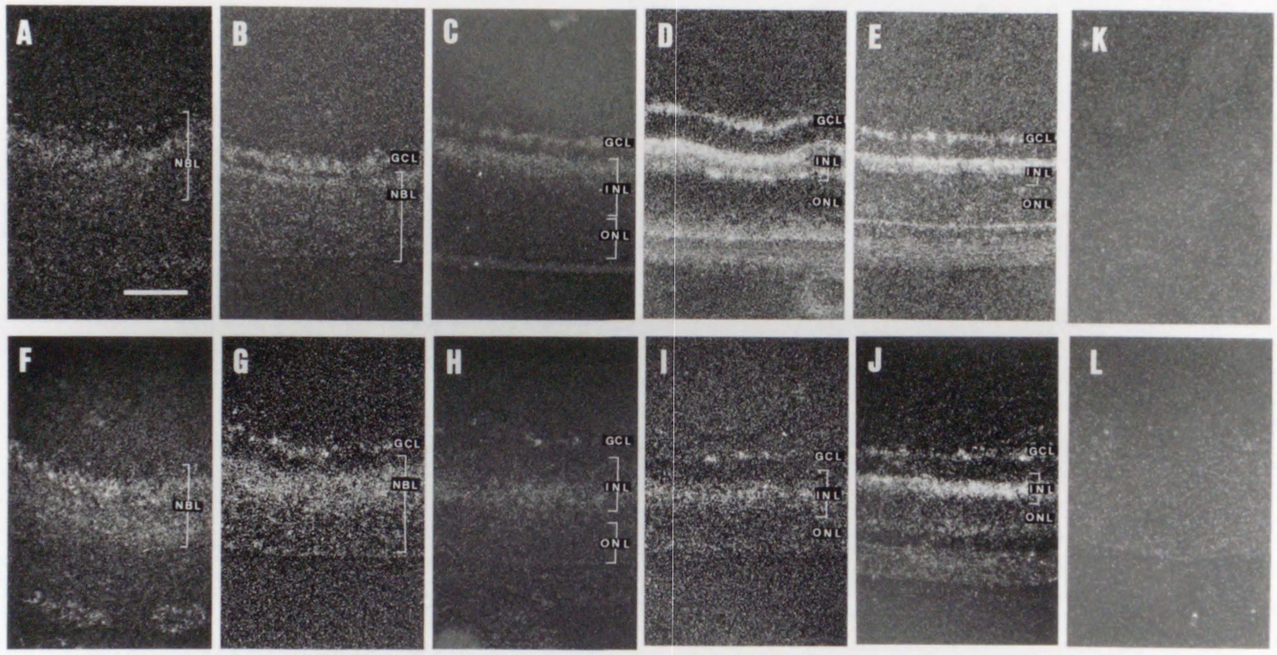
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Figure 1

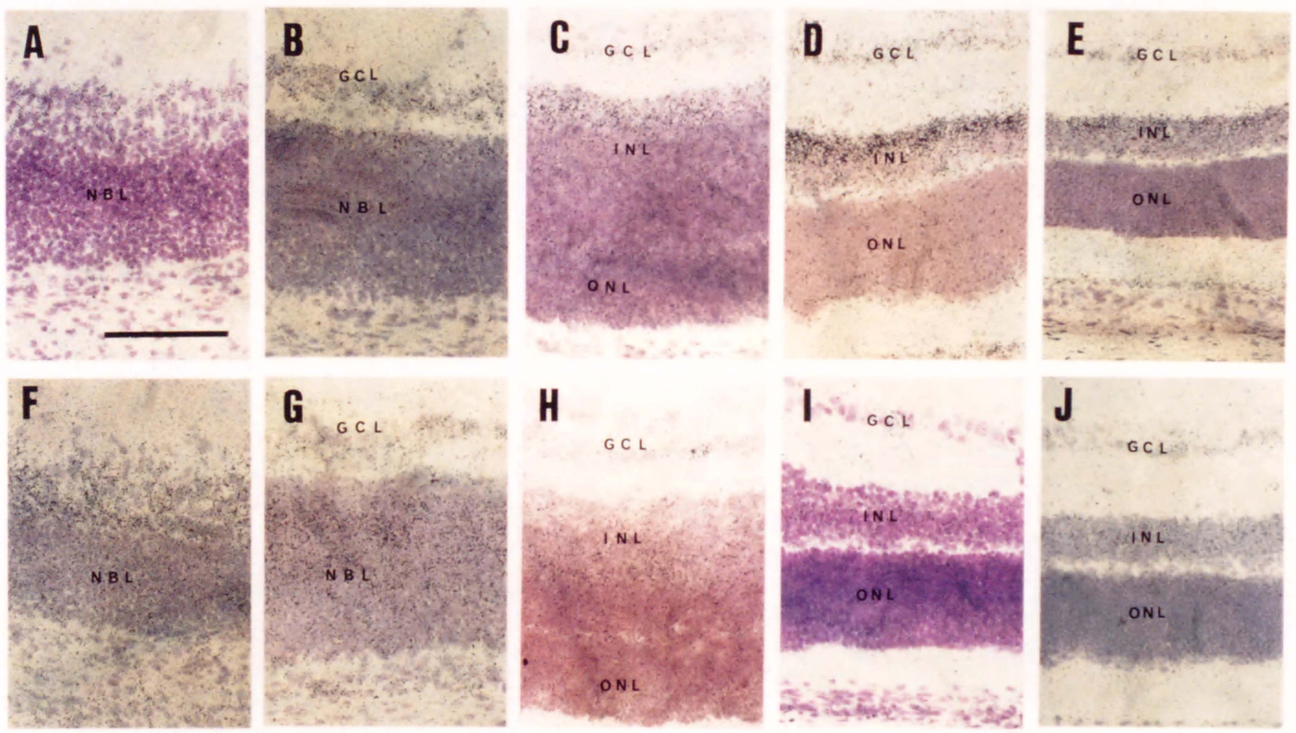
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Figure 2